Eukaryote Diversity in Chicago Area Water Samples

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by

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ABSTRACT

In the past two decades, there has been an increase in the number of *Acanthamoeba* keratitis infections reported both in the United States and globally. *Acanthamoeba* keratitis (AK) is a painful eye infection caused by the protist *Acanthamoeba*, which can result in blindness if not treated in a timely, effective manner. While the greater number of diagnosed cases is partially due to an increased awareness of the disease and its symptoms, geographic and demographic outbreaks have brought the members of the genus *Acanthamoeba* under severe scrutiny in the scientific community.

In the laboratory of Dr. Paul Fuerst, Department of Evolution, Ecology, and Organismal Biology at the Ohio State University, projects are currently active to identify all eukaryotic organisms present in diverse water samples from several locales across the country where the occurrence of AK has been atypically high. The water samples analyzed in this study, obtained from the Chicago area, were screened for further microbial content following positive identification of *Acanthamoeba* in the sample. Similar previous studies of this nature have revealed a myriad of other microorganisms thriving in samples collected from treated water sources. Through the use of precise DNA treatment techniques, specifically PCR amplification of the small subunit rRNA gene followed by DNA sequencing of cloned amplification products, this screen identified the probable presence of the amoeba *Hartmannella* in all six Chicago area water samples with discernable sequence results.

During this study, nine Chicago are water samples were analyzed. Eight contained DNA after the initial extraction and were then cloned to investigate the eukaryotic diversity present. There were a total of 62 readable sequences obtained, with 36 returning identifiable results when entered into NCBI BLAST search. The content of the examined Chicago water samples, beyond *Acanthamoeba*, appears to be predominantly the amoeba *Hartmannella* (97%), although there was one instance where the mold *Amylomyces rouxii* (3%) was identified as a possible sequence match.

ACKNOWLEDGEMENTS

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INTRODUCTION

Acanthamoeba keratitis is a rare opportunistic infection of the cornea. Prior to the 1980's, *Acanthamoeba* keratitis (AK) was considered unimportant, and an almost unheard-off disease. The explosion in the popularity and accessibility to contact-lenses, however, caused the medical community to both remember and respect this often misdiagnosed eye infection. Studies have revealed that the incidence of AK yearly in the mid-1980's was at least 1.65-2.01 cases per million contact-lens wearers (Schaumberg et al., 1999). Diagnosis is typically delayed in cases of *Acanthamoeba* keratitis for a number of reasons. Early infections are commonly treated as bacterial keratitis, often because ophthalmologists do not routinely culture corneal ulcers and rely on treatment with broad-spectrum antibiotics. Even when cultures are performed, they may be negative due to the difficulty in growing *Acanthamoeba* (Sarno and Colby, 2006).

Recently, gene amplification and sequencing techniques, primarily in the variable region of the 18S rDNA, have been developed to target *Acanthamoeba* for diagnosis. These new methods allow in-depth studies concerning the specifics of pathogenicity among those species which have the ability to cause AK, and results in improved detection standards in clinical settings (Schroeder et al., 2001). The gene amplification techniques also allow easier analysis of the amoebic flora that may be associated with the environment that contributes to infections. *Acanthamoeba* can be identified in water samples from the home or contact lens solutions of individuals who have become infected with AK. In complementation to such studies, research on the associated microbial flora of water samples which also contain *Acanthamoeba* has become critical to

ensure a complete investigation of all causes and contributors to infectious eye diseases, such as AK.

WHAT IS *ACANTHAMOEBA* KERATITIS?

Acanthamoeba Keratitis is an often misdiagnosed eye infection caused by the protist *Acanthamoeba*. *Acanthamoeba* infections (AK) usually present with nonspecific symptoms, including redness of the eye, reduced vision, tearing, and photophobia. Although the development of AK begins with redness and eye pain, if untreated or incorrectly treated, AK can form an abscess and penetrate the cornea, requiring the necessity of a corneal transplant or causing total blindness in the affected eye (Seal, 1994). The disease is most common among contact-lens users although cases associated with non-contact wearers have been diagnosed, often in association with some form of eye trauma (Stehr-Green et al., 1989; Tachikawa et al., 1995). In contact-lens users, the possibility for *Acanthamoeba* to gain sustained access to the cornea is greatly increased over non-wearers. Improper cleaning, storage in contaminated lens cases, extended use beyond prescribed life of the lens, and the close interface between the (contaminated) lens and the cornea for an extended time during contact-lens use gives the perfect opportunity for *Acanthamoeba* Keratitis to develop ("*Acanthamoeba* and contact lenses").

The primary concern with *Acanthamoeba* and disease is that *Acanthamoeba* is found virtually everywhere, from soil to diverse liquid environments, and is capable of forming a protective cyst which increases the resistance of the protist to some forms of treatment. The cyst form is resistance to many forms of chemical sterilization, including the low levels of chlorine used to sterilize tap water (Moore et al, 1987). *Acanthamoeba*

trophozoites are resistant to chlorine concentrations up to 2ppm, while cysts are able to withstand concentrations nearing 50ppm; the typical concentration of chlorine in public treated water is <1ppm. Treatment of AK with biguanide antiseptic chlorhexidine or topolyhexamethylene biguanide has recently proven more effective than previous treatments with propamidine and neomycin, however the premise still remains that fast diagnosis and treatment prevents complications and damage associated with advanced stages of AK (Seal). Despite what is known about the effects of *Acanthamoeba* in AK infections, the cause of *Acanthamoeba's* pathogenicity remains unclear. Multiple research teams globally are currently working to solve this mystery and reveal the secret to *Acanthamoeba's* destructive role in AK.

STUDYING THE EUKARYOTIC DIVERSITY IN WATER SAMPLES

Despite treatment of water supplies (using disinfectants, depressed temperatures, and decreased organic carbon levels) intended to purify and prepare the water for human use and consumption, the occurrence of *Acanthamoeba* in collected water samples is not as rare as one would like to believe. Having said this, the presence of other microorganisms in water supplies is well recognized. Many studies have been conducted to identify the presence and diversity of protists in different ecosystems, ranging from deep-sea vents to rivers to farm-animal drinking water (Snelling et al, Slapeta et al).

It is the aim of this study to investigate the eukaryotic diversity of water samples which contained *Acanthamoeba*. In revealing the extent of protists present, the effectiveness of water treatment, and also the resilience of these microbes and frequency of co-occurrence with *Acanthamoeba*, can be assessed and compared qualitatively. To

this end, the use of DNA sequence identification based on the 18S rDNA region of the eukaryotic genome was employed.

THE UTILITY OF 18S rDNA IN PROTIST IDENTIFICATION

Ribosomes, the protein-producing factories of the cell, are composed of ribosomal proteins and rRNA. Each ribosome is comprised of a small and a large subunit, and in eukaryotic cells the small subunit includes the 18S rRNA. In order for the cell to produce enough rRNA's for use in ribosomes, the rRNA genes occur in multiple copies in the genome (*E. coli* have seven copies, and *Xenopus* have 600) (Alberts et al., 2002). The repetitive nature of rDNA makes its use in sequencing identification much more successful compared to genes present in the genome in single copies.

18S rDNA has other unique properties which make it a practical choice for sequence analysis. First, the sequences of the rRNA are conserved to a degree such that "universal" primers can be applied during PCR reactions to amplify all (or at least most) of the eukaryotic rDNA present in a sample. This greatly simplifies the task of screening samples for microbe content: rather than performing multiple PCR reactions, all the while at the risk of missing a protist due to lack of the correct primer, the entire rDNA content of an extracted sample can be amplified in one amplification reaction. At the same time, variation within the 18S rDNA sequence is high enough that identification at the genus and even species level can be completed when the amplification products are cloned and sequenced (Wu et al., 2003).

METHODS

SOURCE OF PRIMERS

The primers used in this experiment were identified in previous work to differentiate rDNA identification regions within the *Acanthamoeba* nuclear and mitochondrial genomes. The primers were chosen as they produce an amplimer (amplified gene sequence region) large enough to provide sufficient and reliable results to differentiate between all known eukaryotic genotypes based on the production of the approximately 1500bp genotype-specific amplimer B1 (GSTA.B1) 18S rDNA fragment. GSTA.B1 includes an *Acanthamoeba* identification sequence site, plus an extended neighboring region of the 18S rDNA that is variable within eukaryotes, and thus is useful for identification purposes in species beyond *Acanthamoeba*. The forward primer used for the amplification of eukaryotic 18S rDNA in the GSTA.B1 sequence was CRN5 (5'-CTGGTTGATCCTGCCAGTAG), and the reverse primer was 1137 (5'-GTGCCCTTCCGTCAAT) (Schroeder et al., 2001).

DNA SAMPLES

The EPA has recently instituted modifications to reduce the concentration of residual biocides (disinfectants and disinfection byproducts) in tap water in the interest of protecting public health (United States, 1998). Since then, outbreaks of AK have been documented in several cities; including Chicago, IL; where a 6-fold increase of diagnosed cases was reported since 2003 (Joslin et al., 2006) . It has been hypothesized that the increase in AK cases is due to this reduction in biocides and so tap water in the Chicago area has been sampled for *Acanthamoeba*.

Water sample collection kits containing sterile swabs, alcohol wipes, and 50ml sterile tubes were sent to cases and controls from a retrospective cohort study by Charlotte Joslin of the Department of Ophthalmology and Visual Sciences at the University of Illinois-Chicago. Participants were instructed to obtain 50mL water and swab a 4 inch square area inside the toilet cistern tank below the water line. Samples were returned to Joslin at UIC and then forwarded to Megan Shoff at the Ohio State University with all identifying information removed. Samples were labeled upon receipt with the form Wsample number-year (i.e. W046-06).

Water samples were initially processed by Megan Shoff for the presence of *Acanthamoeba* and other amoeba using light microscopy. Samples in which amoeba (other than *Acanthamoeba*) were identified were stored at room temperature in amoeba saline until DNA extraction. When a sample was to be extracted for the DNA content of the protists present, the storage plate was scraped with a Disposable Cell Scraper (Costar, Cambridge, MA) to dislodge any protists which had adhered to the plate before the water sample was transferred to the appropriate microcentrifuge tube. Samples were spun down and the supernatant removed. DNA extraction was then performed using the DNeasy® Tissue Kit (QIAGEN Inc., Valencia, California) per manufacturer's instructions for extraction of animal tissues to obtain the DNA.

INITIAL AMPLIFICATION OF DNA

The DNA samples were amplified using polymerase chain reaction (PCR) under conditions appropriate to the specific primers used. Each reaction totaled 25µl comprised of 3µl template, 1µl 2pM Primer 1 (CRN5), 1µl 2pM Primer 2 (1137), 4µl nucleotides,

2.5µl ThermoPol Buffer 10x Concentrate (New England BioLabs Inc., Ipswich, MA), 0.25µl Taq DNA Polymerase (New England BioLabs Inc., Ipswich, MA), and 13.25µl water. The conditions for the reaction were as follows: 5 minutes at 94^{O}C; 40 cycles: (1 minute at 95 ^{O}C; 2 minutes at 56 ^{O}C; 2.5 minutes at 72 ^{O}C); 15 minutes at 72 ^{O}C (hold temperature after completion at 10 ^{O}C).

In cases where the newly extracted DNA did not amplify appropriately with the Taq DNA Polymerase, the PCR was set up again using TITANIUM^{TM} Taq DNA Polymerase and 10x Titanium Taq PCR Buffer (Clonetech Laboratories Inc., Mountain View, CA) and the amplification reaction run again, using the same conditions given above.

CONFIRMATION OF DNA CONTENT

A 50ml agarose gel was prepared for gel electrophoresis using 0.4g SeaKem® GTG Agarose (Cambrex Bio Science Rockland Inc., Rockland, ME), 0.3g Synergel^{TM} Agarose Clarifier Additive (Diversified Biotech, Boston, MA), and 50 ml 0.5xTBE (see Appendix 1 for preparation). The mixture was heated until all solids dissolved and the solution became uniform. Ethidium bromide SpinBind® Reagent C Sodium Iodide Wash Buffer (8µl) (FMC Bioproducts, Rockland, ME) was mixed in, then the solution allowed to cool several minutes before pouring into the template. Samples (7µl) were mixed with 2µl loading dye and added to the wells in the gel; 3µl 1kb Plus DNA Ladder (Invitrogen Corporation**,** Carlsbad, California) was mixed with loading dye and TBE for size reference. The reaction ran at 100V until the first visual marker band had moved down approximately 2/3 the length of the gel.

PURIFICATION OF DNA

The remaining PCR product (18µl) was cleaned using QIAquick® PCR Purification Kit (50) (QIAGEN Inc., Valencia, California) as per manufacturer's instructions.

DNA CLONING

To prepare for the cloning procedure, 1µl cleaned PCR product was diluted with 9µl water. Cloning was performed using the TOPO TA Cloning® Kit pCR® 2.1 TOPO® vector (Invitrogen Corporation**,** Carlsbad, California) according to manufacturer's instructions. (See Appendix 2 for cloning vector).

ELIMINATING FALSE POSITIVES

To confirm the presence of DNA in the clone product, the following mixture was prepared and incubated at 37 ^{O}C for two hours: 5 µL clone final product; 3 µL water; 1 µL ECO R1 (Invitrogen Corporation**,** Carlsbad, California); 1 µL 10x REACT® 3 Buffer (ECO R1 kit; Invitrogen Corporation**,** Carlsbad, California). Samples were then loaded onto a prepared agarose gel and the reaction allowed to run until the first visual marker band had moved approximately 2/3 the length of the gel. The presence of two bands in a lane upon visualization of the DNA indicated the sample did in fact contain an insertion DNA fragment and was therefore suitable for sequencing.

SEQUENCING PREPARATION

The clone samples confirmed to contain DNA inserts were prepared for sequencing amplification via PCR by using conditions revised by Megan Shoff. Each reaction included 1.5µl clone template, 0.5µl M13+ primer (Invitrogen Corporation**,** Carlsbad, California), 1µl ThermoPol Buffer 10x Concentrate (New England BioLabs Inc., Ipswich, MA), 0.5µl BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and 1.5µl water. The reaction conditions were as follows: 25 cycles: (5 seconds at 94 ^{O}C; 10 seconds at 55 ^{O}C; 4 minutes at 60 ^{O}C); hold temperature after completion at 10 ^{O}C. PCR products were then prepared for sequencing using ethanol precipitation (Appendix 3), and sent to the laboratory of H. Lisle Gibbs (370 Aranoff Laboratory, 318 W 12th Ave, Columbus, OH 43210) to be sequenced using a ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were then analyzed using BioEdit Sequence Alignment Editor and NCBI BLAST.

RESULTS

Nine Chicago-Area water samples (samples W012-06, W020-06, W046-06, W047-06, W058-06, W059-06, W060-06, W018-07, and W191-07) of those received from Dr. C. Joslin were analyzed for identification of the protist diversity present. Sample W047-06 did not yield any DNA from the extraction process; while all other samples exhibited some DNA content (refer to Figure 1). Extracted DNA from each sample was cloned and cultured on one of several plates (represented by the letter at the end of the clone name; ex: W048-06A), and individual clones then labeled with a number (ex: W048-06A1). Of 134 clones selected from the culture plate for analysis, 113 (84%) grew and were treated for DNA extraction. The presence of DNA in false positive tests can be observed in Figure 2; 89 (79%) clones contained insertion DNA which was amplified for sequencing. The sequences from these clones, minus any peripheral vector regions, were then analyzed using NCBI-BLAST nucleotide-nucleotide search (non-human, non-mouse database) (Altschul, et al., 1990) to identify the putative organism that they represent. Of the 62 (70% sequencing success) clonal insertions producing readable sequences, 34 (58%) gave identifiable results. The remaining clones presumably represented incomplete fragments and vector sequences.

Table 1 lists the water samples, showing the complete set of clones identified to have insertion DNA sequences, and the most likely identification for each clone. Table 2 gives more detailed information about most probable identity of the sequences which were able to be identified in NCBI BLAST. Clonal DNA sequences were obtained for all samples (excluding W047-06); however, identifiable clones were obtained only from six of the eight samples. Samples W020-06 and W191-07 combined had a total of 10 cloned

sequences, but none of these sequences were identifiable when queried in NCBI BLAST. The diversity of the identifiable sequences found is as follows:

W012-06, *Hartmannella sp*. (5/8) and *Amylomyces rouxii* (1/8);

W046-06, *Hartmannella vermiformis* (1/7);

W058-06, *Hartmannella vermiformis* (3/4);

W059-06, *Hartmannella vermiformis* (8/9);

W060-06, *Hartmannella sp*. (15/19);

and W018-07, *Hartmannella vermiformis* (1/5).

The microorganisms recognized by the BLAST search consisted predominately of *Hartmannella*, which was identified in all six of the water samples producing valid sequences (33 of 34 [97%] clone sequences). Additionally, the mold *Amylomyces rouxii* was the most likely match for clone W012-06B4. Upon closer inspection of the sequences received back from samples containing multiple *Hartmannella* sequences, there appears to be multiple strains present. Further investigation of variaility between these sequences is currently in progress.

DISCUSSION

Based on analysis of clone sequences obtained from DNA extraction of Chicago area water samples, the eukaryotic diversity present in the population most likely contains *Hartmannella veriformis* (amoebozoa) and *Amylomyces rouxii* (mold). The finding of possible *Hartmannella vermiformis* matches is not completely surprising, as this species of amoeba is closely related to the sought-after *Acanthamoeba* also found in these samples (Figure 3).

In total, *Hartmannella* was identifiable in 33 of 34 (97%) sequences containing adequate amounts of genetic code for comparison in NCBI BLAST search, while only 3% (1 sequence) reported A*mylomyces rouxii*. Such results are indicative of a high content of *Hartmennella* in Chicago area water, which is possible, as this protist is a common resident in soil, sewage, and water sources ("Hartmannella" 2004). However, a closer look at the nature of *Hartmannella* and the conditions of the experiment should be taken into account.

During the extraction of samples W046-06, W047-06, W058-06, W059-06, W060-06, a step was inadvertently omitted; culture plates were not scraped with a rubber spatula before the water samples were collected for the initial centrifugation. If *Hartmannella* forms weaker adhesions to its substratum (i.e. the culture plate) compared to the other microorganisms in each sample, it might be captured in the centrifuged fraction of the sample while the other microorganisms remain adhered to the culture plate. This could explain why, if *H. veriformis* were absent, sample W047-06 did not appear to contain any DNA (fig. 1-A) or why all 27 (100%) clones obtained from this set of water samples contained *Hartmannella*. By contrast, only 86% (6/7) clone sequences from the correctly extracted water samples reported the probable identification of *Hartmannella*.

Hartmannella is a slug-like amoeba found in fresh water and potable water systems (Kuchta et al., 1993; MicrobiologyBytes, 2007). This protest is a monopodal pseudopod which can convert to a protective cyst form when environmental conditions are harsh, much like *Acanthamoeba* ("Hartmannella" 2007). Studies have shown that by decreasing the distance between amoebas and the substratum (by increasing levels of electrolytes in the environment), the rate of motility of amoebas can be increased through

improved amoeba-substrate adherence (King, 1979). In contrast, for algae, increasing the electrolyte concentration in the environment results in tighter adherence (diminished motility) to the substratum (Preston and King, 2003). Thus a slight change in the net charge (caused by different combinations and proportions of intracellular proteins and inorganic ions) within the media could affect adherence patterns for different eukaryotes to the culture plate, perhaps allowing *H. veriformis*, almost exclusively, to be released into solution with only the slight agitation of pipetting.

A second explanation is that *H. veriformis* may contain a copy number of the 18S rDNA gene that is much higher than that of the other organisms present and so it is obtained in a higher proportion during PCR amplification and subsequent cloning procedures.

Following these remarks, permitted more time the experimenter would have liked to re-extract the first set of samples (correctly) to identify if and what other eukaryotes are present, and also select more clones to sequence from samples W018-07, W058-06, W020-07 and W191-07.

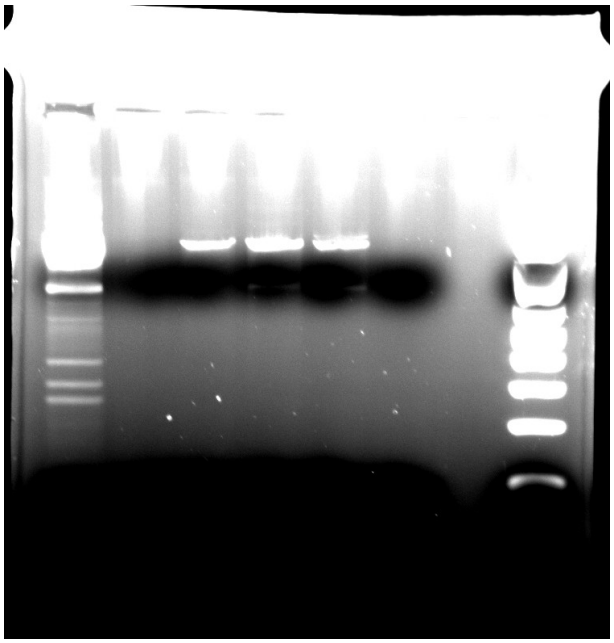
The observation that *Hartmannella* is found in the majority of samples analyzed in this study raises a question about whether *Hartmannella* could be contributing to the increase in *Acanthamoeba* keratitis, or to pathogenic outcomes of other human diseases. Examination of the literature indicates that this question is not resolved for either *Hartmannella* or for other related amoebae (De Jonckheere and Brown, 1998; Kinnear, 2001). There have been reports of isolations of *Hartmannella* and *Vahlkampfia* from human keratitis cases (Kinnear, 2001). Studies have been performed that indicated the cytopathic potential of these isolates in keratocyte tissue cultures (Kinnear, 2003).

However, there is little evidence indicating that these amoebae are pathogenic in animal models and, Koch's Postulates have not been satisfied, since there has been no demonstration of cause and effect for keratitis (De Jonckheere and Brown, 1998). It is known that secondary invasion of lesions or contamination of clinical samples by free-living amoebae can occur. Opportunism by *Hartmannella* may thus explain the association of *Hartmannella* with keratitis, where the keratitis is actually caused by *Acanthamoeba*.

SUMMARY

In conclusion, the identification of eukaryotic diversity in Chicago area water samples was made possible through the application of DNA amplification, cloning, and sequencing techniques. The content of the examined Chicago water samples, beyond *Acanthamoeba*, appears to be predominantly the amoeba *Hartmannella* (97%), although there was an instance where the mold *Amylomyces rouxii* (3%) was identified as a possible sequence match. Several hypotheses exist to explain the high occurrence of *Hartmannella* in the samples examined, however it may be accurate that *Hartmannella* is legitimately present in higher proportions than other eukaryotes in Chicago area water.

(A)



(B)

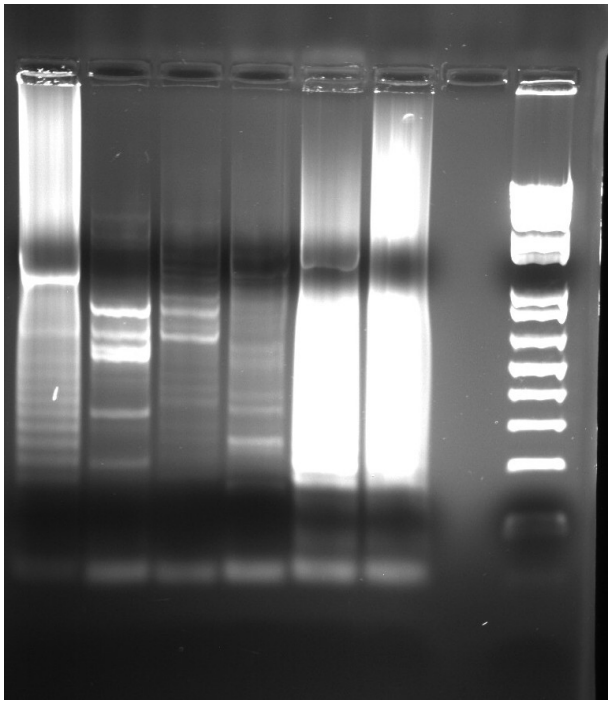
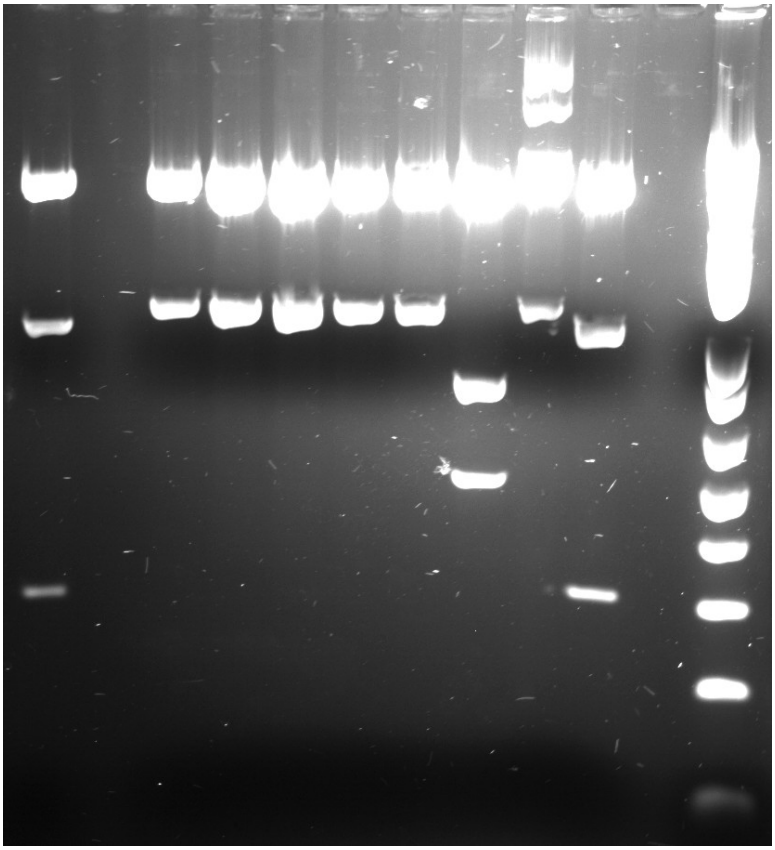


Figure 1. Gel electropherograms of initial DNA extractions. (A) Chicago area water samples W046-06, W047-06, W058-06, W059-06, W060-06; 1kb ladder. All samples except W047-06 contain DNA. (B) Chicago area water samples W012-06, W020-06, W012-07, W191-07; negative, positive, 1kb ladder. All samples contain DNA.

(A)



(B)

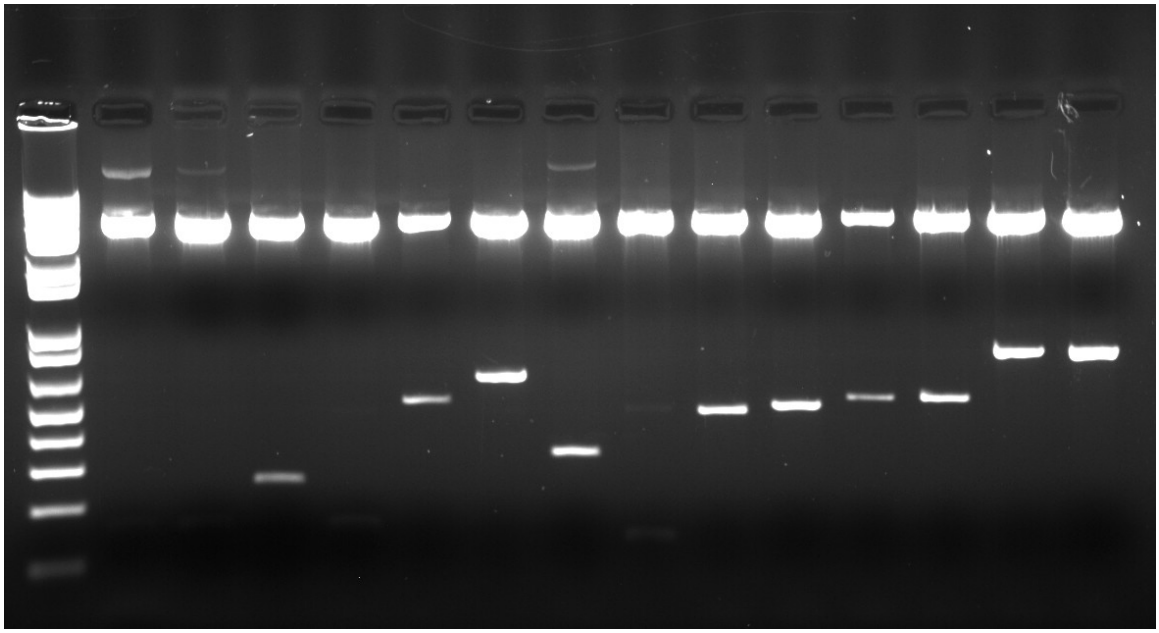


Figure 2. Sample ECO RI digests for identification of false positives. (A) W060-06C1, W060-06C2, W060-06C3, W060-06C4, W060-06C5, W060-06D1, W060-06D2, W060-06D4, W060-06D5; 1kb ladder far right. All clones contain insert DNA sequence. (B) 1kb ladder, W020-06A1, W020-06A2, W020-06A3, W020-06A4, W020-06A5, W191-07A3, W191-07A4, W191-07B1, W191-07B2, W191-07B3, W191-07B4, W191-07B5, and two unknown clones.

Figure 3. Phylogenetic position of *Hartmanella vermiformis* and other members of the lobose amoebae, including *Acanthamoeba* - from: Bolivar et al. (2001)

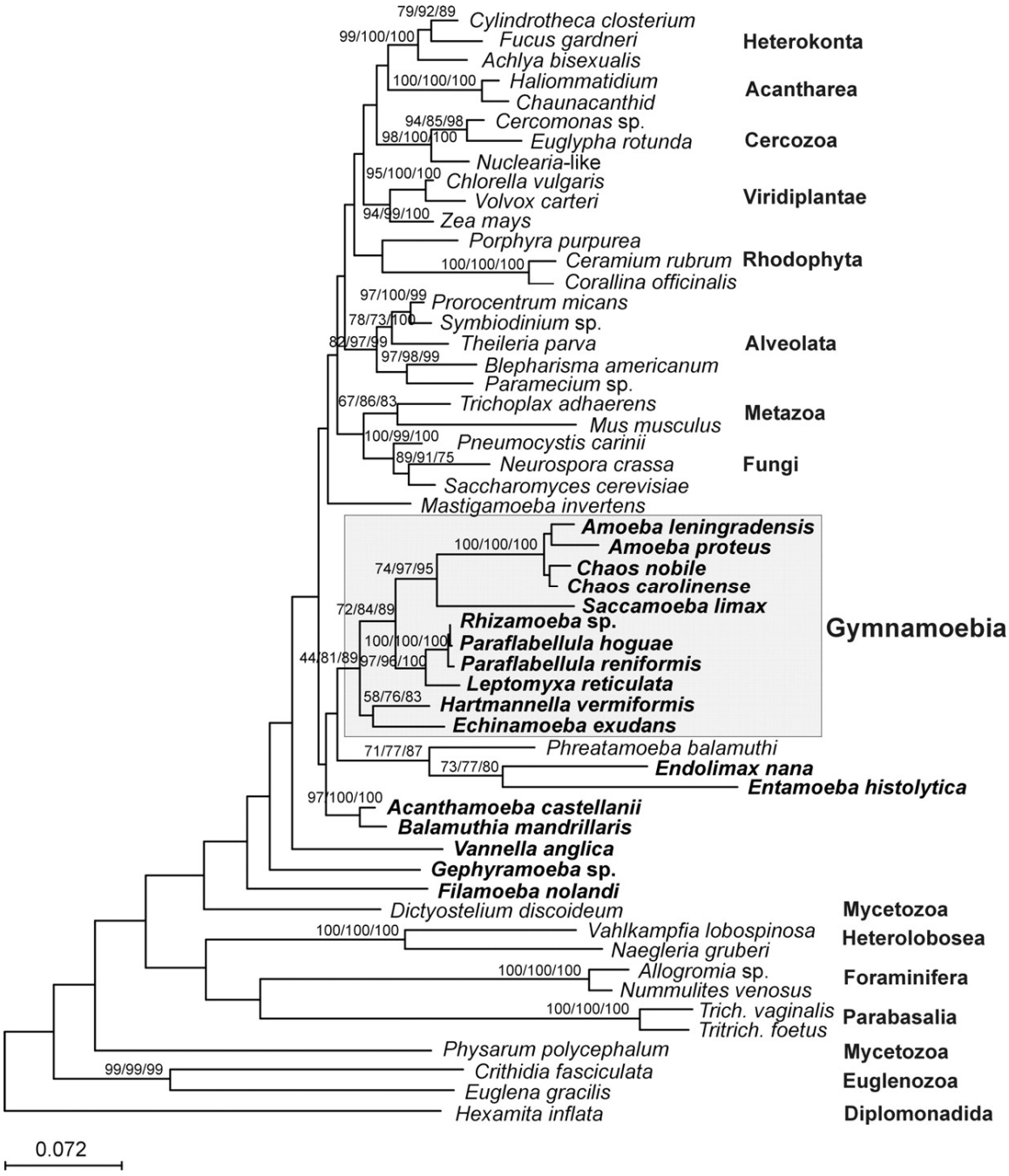


Table 1. Summary of sequence results of clones queried in NCBI BLAST search.

Sample (diversity)	Successfully Grown Clones	Clone Identification
W012-06 (2)	A1	Hartmannella vermiformis
	A3	
	A4	Hartmannella sp.
	A5	Hartmannella sp.
	B1	Hartmannella vermiformis
	B2	Hartmannella sp.
	B4	Amylomyces rouxii
	B5	
W020-06 (0)	A1, A2, A4	
W046-06 (1)	A1, A2, A3	
	A4	Hartmannella vermiformis
	A9, A10	
	B4	
W058-06 (1)	A5	
	A8	Hartmannella vermiformis
	A9	Hartmannella vermiformis
	B6	Hartmannella vermiformis
W059-06 (1)	A1	Hartmannella vermiformis
	A2	Hartmannella vermiformis
	A3	Hartmannella vermiformis
	A4	Hartmannella vermiformis
	A5	Hartmannella vermiformis
	A6	Hartmannella vermiformis
	A7	Hartmannella vermiformis
	A8	Hartmannella vermiformis
	B6	
W060-06 (1)	B2	Hartmannella vermiformis
	C1	Hartmannella vermiformis
	C2	Hartmannella vermiformis
	C3	Hartmannella vermiformis
	C4	Hartmannella vermiformis
	C5	Hartmannella vermiformis
	C6	Hartmannella vermiformis
	C7	Hartmannella vermiformis
	C8	Hartmannella vermiformis
	C9	
	D1	Hartmannella vermiformis
	D2	
	D4	Hartmannella vermiformis
	D5	Hartmannella sp.
	D6	
	D7	Hartmannella vermiformis
	D8	
	D9	Hartmannella vermiformis
	D10	Hartmannella vermiformis
W018-07 (1)	A4	
	A5	Hartmannella vermiformis
	B3, B4, B5	
W191-07 (0)	A1, A2, A3	
	B2, B3, B4, B5	

Table 2. Description of clones identified in NCBI BLAST search.

Clone Name	Species	Direction of sequence	Sequence length (num. bases)	Query Coverage (%)	Maximum Identity (%)
W012-06A1	Hartmannella vermiformis	FR	780	88	97
W012-06A4	Hartmannella sp.	FR	798	73	81
W012-06A5	Hartmennella sp.	FR	657	73	81
W012-06B1	Hartmannella vermiformis	FR	648	75	98
W012-06B2	Hartmannella sp.	RV	672	53-62	85-94
W012-06B4	Amylomyces rouxii	FR	419	70-72	98
W046-06A4	Hartmannella vermiformis	FR	598	89	96-97
W058-06A8	Hartmannella vermiformis	FR	312	98	94-95
W058-06A9	Hartmannella vermiformis	FR	348	94	96
W058-06B6	Hartmannella vermiformis	FR	418	98	98
W059-06A1	Hartmannella vermiformis	FR	782	83-86	98
W059-06A2	Hartmannella vermiformis	FR	562	52	98-99
W059-06A3	Hartmannella vermiformis	FR	612	93	95
W059-06A4	Hartmannella vermiformis	FR	807	85	97-98
W059-06A5	Hartmannella vermiformis	FR	339	30	98-99
W059-06A6	Hartmannella vermiformis	FR	562	74-75	97-98
W059-06A7	Hartmannella vermiformis	FR	827	75-82	95-96
W059-06A8	Hartmannella vermiformis	FR	502	95	98
W060-06B2	Hartmannella vermiformis	RV	737	82	97
W060-06C1	Hartmannella sp. (manual sequence inspection)	FR	350		
W060-06C2	Hartmannella vermiformis	FR	554	91-97	97-98
W060-06C3	Hartmannella vermiformis	RV	763	75-98	96-99
W060-06C4	Hartmannella sp.	FR	336	69	86
W060-06C5	Hartmannella vermiformis	RV	787	74	97-98
W060-06C6	Hartmannella vermiformis	FR	233	81	91-92
W060-06C7	Hartmannella vermiformis	FR	403	73	96

W060-06C8	Hartmannella vermiformis	FR	483	95	98-99
W060-06D1	Hartmannella vermiformis	FR	897	71-72	97
W060-06D4	Hartmannella vermiformis	FR	331	98	97-98
W060-06D5	Hartmannella sp.	FR	752	42	98
W060-06D7	Hartmannella vermiformis	FR	384	98	98-99
W060-06D9	Hartmannella vermiformis	RV	300	23-29	92-94
W060-06D10	Hartmannella vermiformis	FR	592	97	96
W018-07A5	Hartmannella vermiformis	FR	833	83	98

Appendix 1

Preparation of 10xTBE Stock:

In a 2L flask combine:

168 g Tris Base (Invitrogen Corporation**,** Carlsbad, California)
55g Boric Acid (Electrophoresis Grade) (Fisher Scientific)
7.45g disodium ethylenediamine tetraacetate (reagent grade) (Fisher Scientific)
1000ml water.

Add a stir bar and heat gently while stirring until solution becomes clear.

Preparation of 0.5xTBE:

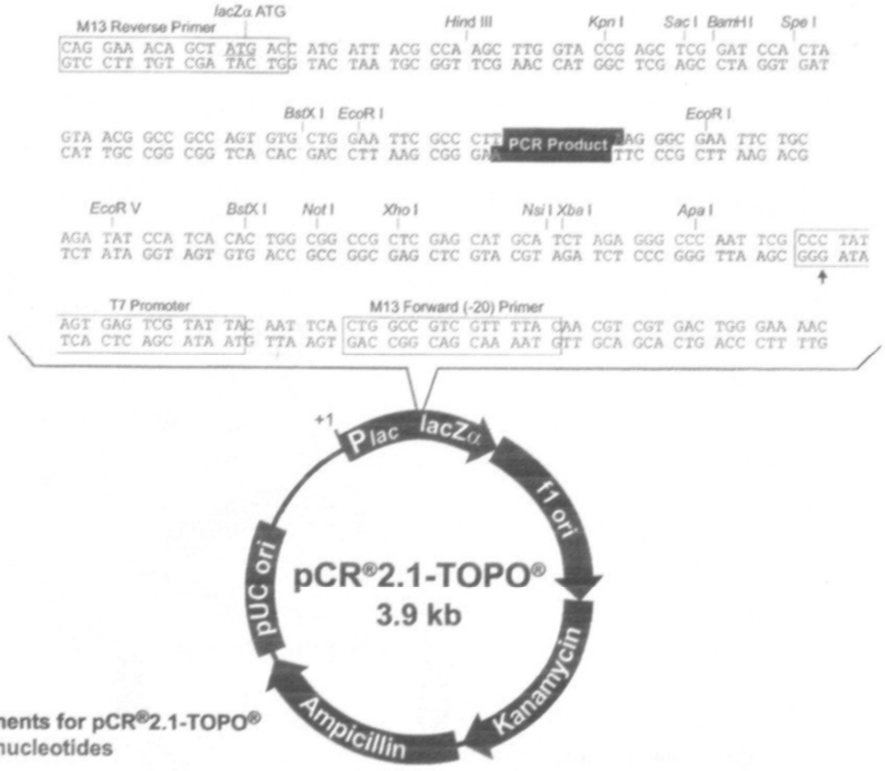
In a large storage container combine 200ml 10xTBE Stock and 3800ml water.

Appendix 2

Map of pCR®2.1-TOPO®

pCR®2.1-TOPO® Map

The map below shows the features of pCR®2.1-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrow indicates the start of transcription for T7 polymerase. **The complete sequence of pCR®2.1-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 24).**



Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Appendix 3

Ethanol Precipitation (for Half Reactions)

1. Remove caps from tubes and:
    a.) 8µl of deionized water
    b.) 32µl of non-denatured 95% ethanol and aspirate
2. Place in a 1.5ml tube
3. Vortex briefly
4. Sit at room temperature for 10 minutes
5. Spin at 13,000 RPM for 20 minutes
6. Remove supernatant with pipette
7. Add 250µl of 70% ethanol
8. Vortex briefly
9. Spin for 10 minutes at 13,000 RPM
10. Gently poor off supernatant and remove any remaining supernatant with pipette
11. Dry samples for 30 minutes in a 70 ^{O}C hot water bath.
12. Add 15µl Hi-Di^{TM} Formamide (Applied Biosystems, Foster City, CA)
13. Aspirate several times while scraping the bottom of the tube
14. Vortex for 5 seconds
15. Spin down
16. Transfer to sequencing tubes

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